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(71) Applicant: THE PROCTER & GAMBLE COMPANY [US/US]; One Procter & Gamble Plaza, Cincinnati, OH 45202 (US).

(72) Inventors: BRODE, Philip, Frederick, III; 5780 Squirrelnest Lane, Cincinnati, OH 45252 (US). BARNETT, Bobby, Lee; 12175 Elkwood Drive, Cincinnati, OH 45240 (US). RUBINGH, Donn, Nelton; 8224 Sheed Road, Cincinnati, OH 45247 (US). GHOSH, Chanchal, Kumar; 7005 Pinemill Drive, West Chester, OH 45069 (US).

(74) Agents: REED, T., David et al.; The Procter & Gamble Company, 5299 Spring Grove Avenue, Cincinnati, OH 45217 (US).

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(54) Title: FABRIC CLEANING COMPOSITIONS CONTAINING SUBTILISIN BPN' VARIANTS

(57) Abstract

The present invention relates to fabric cleaning compositions comprising subtilisin BPN' variants, wherein the BPN' variant comprises one or more amino acid positions having a different amino acid than that occurring in wild-type subtilisin BPN' (i.e., substitution) at specifically identified positions, whereby the BPN' variant has decreased adsorption to, and increased hydrolysis of, an insoluble substrate as compared to wild-type subtilisin BPN'.

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FABRIC CLEANING COMPOSITIONS CONTAINING SUBTILISIN BPN' VARIANTS

TECHNICAL FIELD

The present invention relates to fabric cleaning compositions comprising protease enzymes which are subtilisin variants.

BACKGROUND

Enzymes make up the largest class of naturally occurring proteins. Each class of enzyme generally catalyzes (accelerates a reaction without being consumed) a different kind of chemical reaction. One class of enzymes known as proteases, are known for their ability to hydrolyze (break down a compound into two or more simpler compounds with the uptake of the H and OH parts of a water molecule on either side of the chemical bond cleaved) other proteins. This ability to hydrolyze proteins has been taken advantage of by incorporating naturally occurring and protein engineered proteases as an additive to laundry detergent preparations. Many stains on clothes are proteinaceous and wide-specificity proteases can substantially improve removal of such stains.

Unfortunately, the efficacy level of these proteins in their natural, bacterial environment, frequently does not translate into the relatively unnatural wash environment. Specifically, protease characteristics such as thermal stability, pH stability, oxidative stability and substrate specificity are not necessarily optimized for utilization outside the natural environment of the enzyme.

The amino acid sequence of the protease determines the characteristics of the protease. A change of the amino acid sequence of the protease may alter the properties of the enzyme to varying degrees, or may even inactivate the enzyme, depending upon the location, nature and/or magnitude of the change in the amino acid sequence. Several approaches have been taken to alter the wild-type amino acid sequence of proteases in an attempt to improve their properties, with the goal of increasing the efficacy of the protease in the wash environment. These approaches include altering the amino acid sequence to enhance thermal stability and to improve oxidation stability under quite diverse conditions.

Despite the variety of approaches described in the art, there is a continuing need for compositions comprising effective variants of proteases useful for cleaning fabric surfaces.

Objects of the Present Invention

It is an object of the present invention to provide fabric cleaning compositions comprising subtilisin enzyme variants.

SUMMARY

The present invention relates to compositions comprising subtilisin BPN' variants for cleaning fabric surfaces. The BPN' variants useful in these compositions comprise at least one, two or three amino acid positions having a different amino acid than that occurring in wild-type subtilisin BPN' (i.e., substitution) at specifically identified positions, whereby the BPN' variant has decreased adsorption to, and increased hydrolysis of, an insoluble substrate as compared to the wild-type subtilisin BPN'.

DESCRIPTION

I. Subtilisin Variants Useful In Fabric Cleaning Compositions

This invention relates to fabric cleaning compositions comprising a subtilisin enzyme, in particular BPN', that has been modified by mutating the various nucleotide sequences that code for the enzyme, thereby modifying the amino acid sequence of the enzyme. The modified subtilisin enzymes (hereinafter, "BPN' variants") useful in the compositions of the present invention have decreased adsorption to and increased hydrolysis of an insoluble substrate as compared to the wild-type subtilisin. Certain of these BPN' variants are described in co-pending application U.S.S.N. 08/121,437, filed September 15, 1993 by Brode et al.

The subtilisin enzymes useful in the compositions of this invention belong to a class of enzymes known as proteases. A protease is a catalyst for the cleavage of peptide bonds. One type of protease is a serine protease. A serine protease is distinguished by the fact that there is an essential serine residue at the active site.

The observation that an enzyme's rate of hydrolysis of soluble substrates increases with enzyme concentration is well documented. It would therefore seem plausible that for surface bound substrates, such as is encountered in many cleaning applications, the rate of hydrolysis would increase with increasing surface concentration. This has been shown to be the case. (Brode, P.F. III and D. S. Rauch, LANGMUIR, "Subtilisin BPN': Activity on an Immobilized Substrate", Vol. 8, pp. 1325-1329 (1992)). In fact, a linear dependence of rate upon surface concentration was found for insoluble substrates when the surface concentration of the enzyme was varied. (Rubingh, D. N. and M. D. Bauer, "Catalysis of Hydrolysis by Proteases at the Protein-Solution Interface," in POLYMER SOLUTIONS, BLENDS

AND INTERFACES, Ed. by I. Noda and D. N. Rubingh, Elsevier, p. 464 (1992)). Surprisingly, when seeking to apply this principle in the search for variant proteases which give better fabric cleaning performance, we did not find that enzymes which adsorb more give better performance. In fact, we surprisingly determined the opposite to be the case: decreased adsorption by an enzyme to a substrate resulted in increased hydrolysis of the substrate (i.e., better cleaning performance).

While not wishing to be bound by theory, it is believed that improved performance, when comparing one variant to another, is a result of the fact that enzymes which adsorb less are also less tightly bound and therefore more highly mobile on the surface from which the insoluble protein substrate is to be removed. At comparable enzyme solution concentrations, this increased mobility is sufficient to outweigh any advantage that is conferred by delivering a higher concentration of enzyme to the surface.

The mutations described herein are designed to change (i.e., decrease) the adsorption of the enzyme to surface-bound soils. In BPN', the amino acids from position 199 to position 220 form a large exterior loop on the enzyme molecule. It has been discovered that this loop plays a significant role in the adsorption of the enzyme molecule to a surface-bound peptide, and specific mutations in this loop have a significant effect on this adsorption. While not wishing to be bound by theory, it is believed that this loop is important to the adsorption of the BPN' molecule for at least two reasons. First, the amino acids which comprise this exterior loop can make close contacts with any surfaces to which the molecule is exposed. Second, the proximity of this loop to the active-site and binding pocket of the BPN' molecule gives it a role in the catalytically productive adsorption of the enzyme to surface-bound substrates (peptides/protein soils).

As used herein, "variant" means an enzyme having an amino acid sequence which differs from that of wild-type.

As used herein, "mutant BPN' gene" means a gene coding for a BPN' variant.

As used herein, "wild-type subtilisin BPN'" refers to a subtilisin enzyme represented by SEQ ID NO:1. The amino acid sequence for subtilisin BPN' is further described by Wells, J. A., E. Ferrari, D. J. Henner, D. A. Estell and E. Y. Chen, NUCLEIC ACIDS RESEARCH, Vol. II, 7911-7925 (1983), incorporated herein by reference.

As used herein, the term "wild-type amino acid sequence" encompasses SEQ ID NO:1 as well as SEQ ID NO:1 having modifications to the amino acid sequence other than at any of positions 199-220.

As used herein, "more hydrophilic amino acid" refers to any other amino acid having greater hydrophilicity than a subject amino acid with reference to the hydrophilicity table below. The following hydrophilicity table (Table 1) lists amino acids in descending order of increasing hydrophilicity (see Hopp, T.P., and Woods, K.R., "Prediction of Protein Antigenic Determinants from Amino Acid Sequences", PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCE USA, Vol. 78, pp. 3824-3828, 1981, incorporated herein by reference).

TABLE 1

Amino Acid	Hydrophilicity Value
Trp	-3.4
Phe	-2.5
Tyr	-2.3
Leu, Ile	-1.8
Val	-1.5
Met	-1.3
Cys	-1.0
Ala, His	-0.5
Thr	-0.4
Pro, Gly	-0.0
Gln, Asn	0.2
Ser	0.3
Arg^+, Lys^+, Glu^-, Asp^-	3.0

Table 1 also indicates which amino acids carry a charge (this characteristic being based on a pH of from about 8-9). The positively charged amino acids are Arg and Lys, the negatively charged amino acids are Glu and Asp, and the remaining amino acids are neutral. In a preferred embodiment of the present invention, the substituting amino acid is either neutral or negatively charged, more preferably negatively charged (i.e., Glu or Asp).

Therefore, for example, the statement "substitute Gln with an equally or more hydrophilic amino acid which is neutral or has a negative charge" means Gln would be substituted with Asn (which is equally hydrophilic to Gln), or Ser, Glu or Asp (which are more hydrophilic than Gln); each of which are neutral or have a negative charge, and have a greater hydrophilicity value as compared to Gln. Likewise, the statement "substitute

Pro with a more hydrophilic amino acid which is neutral or has a negative charge" means Pro would be substituted with Gln, Asn, Ser, Glu or Asp.

A. Variants comprising at least one amino acid substitution

In one embodiment of the present invention, the BPN' variant comprises wild-type amino acid sequence wherein the wild-type amino acid sequence at one or more of positions 199, 200, 201, 202, 203, 204, 205, 206, 207, 208, 209, 210, 211, 212, 213, 214, 215, 216, 218, 219 or 220 is substituted; whereby the BPN' variant has decreased adsorption to, and increased hydrolysis of, an insoluble substrate as compared to the wild-type subtilisin BPN'. Preferably, the positions having a substituted amino acid are 199, 200, 201, 202, 205, 207, 208, 209, 210, 211, 212 or 215; more preferably, 200, 201, 202, 205 or 207.

Preferably, the substituting amino acid for position 199 is Cys, Ala, His, Thr, Pro, Gly, Gln, Asn, Ser, Asp or Glu.

Preferably, the substituting amino acid for position 200 is His, Thr, Pro, Gly, Gln, Asn, Ser, Asp or Glu.

Preferably, the substituting amino acid for position 201 is Gly, Gln, Asn, Ser, Asp or Glu.

Preferably, the substituting amino acid for position 202 is Pro, Gln, Asn, Ser, Asp or Glu.

Preferably, the substituting amino acid for position 203 is Met, Cys, His, Pro, Gly, Gln, Asn, Ser, Asp or Glu.

Preferably, the substituting amino acid for position 204 is Glu.

Preferably, the substituting amino acid for position 205 is Leu, Met, Cys, Ala, His, Thr, Pro, Gly, Gln, Asn, Ser, Asp or Glu.

Preferably, the substituting amino acid for position 206 Pro, Asn or Ser.

Preferably, the substituting amino acid for position 207 is Asp or Glu.

Preferably, the substituting amino acid for position 208 is Pro, Gly, Gln, Asn, Ser, Asp or Glu.

Preferably, the substituting amino acid for position 209 is Ile, Val, Met, Cys, Ala, His, Thr, Pro, Gly, Gln, Asn, Ser, Asp or Glu.

Preferably, the substituting amino acid for position 210 is Gly, Gln, Asn, Ser, Asp or Glu.

Preferably, the substituting amino acid for position 211 is Ala, Pro, Gln, Asn, Ser, Asp or Glu.

Preferably, the substituting amino acid for position 212 is Gln, Ser, Asp or Glu.

Preferably, the substituting amino acid for position 213 is Trp, Phe, Tyr, Leu, Ile, Val, Met, Cys, Ala, His, Pro, Gly, Gln, Asn, Ser, Asp or Glu.

Preferably, the substituting amino acid for position 214 is Phe, Leu, Ile, Val, Met, Cys, Ala, His, Pro, Gly, Gln, Asn, Asp or Glu.

Preferably, the substituting amino acid for position 215 is Thr, Pro, Gln, Asn, Ser, Asp or Glu.

Preferably, the substituting amino acid for position 216 is His, Thr, Pro, Gly, Gln, Asn, Ser, Asp or Glu.

Preferably, the substituting amino acid for position 218 is Glu.

Preferably, the substituting amino acid for position 219 is Pro, Gln, Asn, Ser, Asp or Glu.

Preferably, the substituting amino acid for position 220 is Pro, Gly, Gln, Asn, Asp or Glu.

More preferably, the substituting amino acid for any of positions 199, 200, 201, 202, 203, 205, 207, 208, 209, 210, 211, 212, 213, 214, 215, 216, 219 and 220 is, with reference to Table 1, is neutral or negatively charged and equally or more hydrophilic, preferably more hydrophilic, than the amino acid at the subject position in wild-type subtilisin BPN'.

More preferably still, the substituting amino acid for any of positions 199, 200, 201, 202, 203, 205, 207, 208, 209, 210, 211, 212, 213, 214, 215, 216, 219 and 220 is Asp, or Glu; and the substituting amino acid for positions 204 or 218 is Glu.

B. Variants comprising at least two amino acid substitutions

In another embodiment of the present invention, the BPN' variant comprises wild-type amino acid sequence wherein the wild-type amino acid sequence at two or more of positions 199, 200, 201, 202, 203, 204, 205, 206, 207, 208, 209, 210, 211, 212, 213, 214, 215, 216, 217, 218, 219 or 220 is substituted; whereby the BPN' variant has decreased adsorption to, and increased hydrolysis of, an insoluble substrate as compared to wild-type subtilisin BPN'. Preferably, the positions having a substituting amino acid are 199, 200, 201, 202, 205, 207, 208, 209, 210, 211, 212, or 215; more preferably, positions 200, 201, 202, 205 or 207.

Preferably, the substituting amino acid for position 199 is Cys, Ala, His, Thr, Pro, Gly, Gln, Asn, Ser, Asp or Glu.

Preferably, the substituting amino acid for position 200 is His, Thr, Pro, Gly, Gln, Asn, Ser, Asp or Glu.

Preferably, the substituting amino acid for position 201 is Gly, Gln, Asn, Ser, Asp or Glu.

Preferably, the substituting amino acid for position 202 is Pro, Gln, Asn, Ser, Asp or Glu.

Preferably, the substituting amino acid for position 203 is Met, Cys, Ala, His, Thr, Pro, Gly, Gln, Asn, Ser, Asp or Glu.

Preferably, the substituting amino acid for position 204 is Asp or Glu.

Preferably, the substituting amino acid for position 205 is Leu, Val, Met, Cys, Ala, His, Thr, Pro, Gly, Gln, Asn, Ser, Asp or Glu.

Preferably, the substituting amino acid for position 206 is Pro, Asn, Ser, Asp, or Glu.

Preferably, the substituting amino acid for position 207 is Asp or Glu.

Preferably, the substituting amino acid for position 208 is Pro, Gly, Gln, Asn, Ser, Asp or Glu.

Preferably, the substituting amino acid for position 209 is Ile, Val, Met, Cys, Ala, His, Thr, Pro, Gly, Gln, Asn, Ser, Asp or Glu.

Preferably, the substituting amino acid for position 210 is Ala, Gly, Gln, Asn, Ser, Asp or Glu.

Preferably, the substituting amino acid for position 211 is Ala, Pro, Gln, Asn, Ser, Asp or Glu.

Preferably, the substituting amino acid for position 212 is Gln, Ser, Asp or Glu.

Preferably, the substituting amino acid for position 213 is Trp, Phe, Tyr, Leu, Ile, Val, Met, Cys, Ala, His, Thr, Pro, Gly, Gln, Asn, Ser, or Asp.

Preferably, the substituting amino acid for position 214 is Phe, Leu, Ile, Val, Met, Cys, Ala, His, Thr, Pro, Gly, Gln, Asn, Ser, Asp or Glu.

Preferably, the substituting amino acid for position 215 is Thr, Pro, Gln, Asn, Ser, Asp or Glu.

Preferably, the substituting amino acid for position 216 is His, Thr, Pro, Gly, Gln, Asn, Ser, Asp or Glu.

Preferably, the substituting amino acid for position 217 is Leu, Ile, Val, Met, Cys, Ala, His, Thr, Pro, Gly, Gln, Asn, Ser, Asp or Glu.

Preferably, the substituting amino acid for position 218 is Gln, Ser, Asp or Glu.

Preferably, the substituting amino acid for position 219 is Pro, Gln, Asn, Ser, Asp or Glu.

Preferably, the substituting amino acid for position 220 is Pro, Gly, Gln, Asn, Ser, Asp or Glu.

More preferably, the substituting amino acid for any of positions 199, 200, 201, 202, 203, 204, 205, 206, 207, 208, 209, 210, 211, 212, 213, 214,

215, 216, 217, 218, 219 or 220 is, with reference to Table 1, is neutral or negatively charged and equally or more hydrophilic, preferably more hydrophilic, than the amino acid at the subject position in wild-type subtilisin BPN'.

More preferably still, the substituting amino acid for any of positions 199, 200, 201, 202, 203, 204, 205, 206, 207, 208, 209, 210, 211, 212, 214, 215, 216, 218, 219 or 220 is Asp and Glu; for position 217 is Leu, Asp, or Glu; and for position 213 is Asp.

C. Variants comprising at least three amino acid substitutions

In another embodiment of the present invention, the BPN' variant comprises wild-type amino acid sequence wherein the wild-type amino acid sequence of three or more of positions 199, 200, 201, 202, 203, 204, 205, 206, 207, 208, 209, 210, 211, 212, 213, 214, 215, 216, 217, 218, 219 and 220, is substituted; whereby the BPN' variant has decreased adsorption to, and increased hydrolysis of, an insoluble substrate as compared to wild-type subtilisin BPN'. Preferably, the positions having a substituting amino acid are 199, 200, 201, 202, 205, 207, 208, 209, 210, 211, 212, or 215; more preferably positions 200, 201, 202, 205 or 207.

Preferably, the substituting amino acid for position 199 is Cys, Ala, His, Thr, Pro, Gly, Gln, Asn, Ser, Asp or Glu.

Preferably, the substituting amino acid for position 200 is His, Thr, Pro, Gly, Gln, Asn, Ser, Asp or Glu.

Preferably, the substituting amino acid for position 201 is Gly, Gln, Asn, Ser, Asp or Glu.

Preferably, the substituting amino acid for position 202 is Pro, Gln, Asn, Ser, Asp or Glu.

Preferably, the substituting amino acid for position 203 Met, Cys, Ala, His, Thr, Pro, Gly, Gln, Asn, Ser, Asp or Glu.

Preferably, the substituting amino acid for position 204 is selected from the group consisting of Asp or Glu.

Preferably, the substituting amino acid for position 205 is Leu, Val, Met, Cys, Ala, His, Thr, Pro, Gly, Gln, Asn, Ser, Asp or Glu.

Preferably, the substituting amino acid for position 206 is Pro, Asn, Ser, Asp, or Glu.

Preferably, the substituting amino acid for position 207 is Asp or Glu.

Preferably, the substituting amino acid for position 208 is Pro, Gly, Gln, Asn, Ser, Asp or Glu.

Preferably, the substituting amino acid for position 209 is Ile, Val, Met, Cys, Ala, His, Thr, Pro, Gly, Gln, Asn, Ser, Asp or Glu.

Preferably, the substituting amino acid for position 210 is Ala, Gly, Gln, Asn, Ser, Asp or Glu.

Preferably, the substituting amino acid for position 211 is Ala, Pro, Gln, Asn, Ser, Asp or Glu.

Preferably, the substituting amino acid for position 212 is Gln, Ser, Asp or Glu.

Preferably, the substituting amino acid for position 213 is Trp, Phe, Tyr, Leu, Ile, Val, Met, Cys, Ala, His, Thr, Pro, Gly, Gln, Asn, Ser, Asp or Glu.

Preferably, the substituting amino acid for position 214 is Phe, Leu, Ile, Val, Met, Cys, Ala, His, Thr, Pro, Gly, Gln, Asn, Ser, Asp or Glu.

Preferably, the substituting amino acid for position 215 is Thr, Pro, Gln, Asn, Ser, Asp or Glu.

Preferably, the substituting amino acid for position 216 is His, Thr, Pro, Gly, Gln, Asn, Ser, Asp or Glu.

Preferably, the substituting amino acid for position 217 is Leu, Ile, Val, Met, Cys, Ala, His, Thr, Pro, Gly, Gln, Asn, Ser, Asp or Glu.

Preferably, the substituting amino acid for position 218 is Gln, Ser, Asp or Glu.

Preferably, the substituting amino acid for position 219 is Pro, Gln, Asn, Ser, Asp or Glu.

Preferably, the substituting amino acid for position 220 is Pro, Gly, Gln, Asn, Ser Asp or Glu.

More preferably, the substituting amino acid for any of positions 199, 200, 201, 202, 203, 205, 206, 207, 208, 209, 210, 211, 212, 213, 214, 215, 216, 217, 218, 219 or 220 is, with reference to Table 1, is neutral or negatively charged and equally or more hydrophilic, preferably more hydrophilic, than the amino acid at the subject position in wild-type subtilisin BPN'.

More preferably still, the substituting amino acid for any of positions 199, 200, 201, 202, 203, 204, 205, 206, 207, 208, 209, 210, 211, 212, 214, 215, 216, 218, 219 or 220 is Asp or Glu; for position 217 is Leu, Asp, or Glu; and for position 213 is Asp.

D. Preparation of enzyme variants

Example 1

Mutant BPN' Genes

A phagemid (pSS-5) containing the wild type subtilisin BPN' gene (Mitchinson, C. and J. A. Wells, (1989), "Protein Engineering of Disulfide Bonds in Subtilisin BPN', BIOCHEMISTRY, Vol. 28, pp. 4807-4815) is transformed into *Escherichia coli ung*-strain CJ236 and a single stranded uracil-containing DNA template is produced using the VCSM13 helper phage (Kunkel, T.A., J.D. Roberts and R.A. Zakour, "Rapid and efficient site-specific mutagenesis without phenotypic selection", METHODS IN ENZYMOLOGY, Vol. 154, pp. 367-382, (1987); as modified by Yuckenberg, P.D., F. Witney, J. Geisselsoder and J. McClary, "Site-directed in vitro mutagenesis using uracil-containing DNA and phagemid vectors", DIRECTED MUTAGENESIS - A PRACTICAL APPROACH, ed. M.J. McPherson, pp. 27-48, (1991); both of which are incorporated herein by reference). A single primer site-directed mutagenesis modification of the method of Zoller and Smith (Zoller, M.J., and M. Smith, "Oligonucleotide-directed mutagenesis using M13-derived vectors: an efficient and general procedure for the production of point mutations in any fragment of DNA", NUCLEIC ACIDS RESEARCH, Vol. 10, pp. 6487-6500, (1982), incorporated herein by reference) is used to produce all mutants (basically as presented by Yuckenberg, *et al.*, 1991, above). Oligonucleotides are made using an Applied Biosystem Inc. 380B DNA synthesizer. Mutagenesis reaction products are transformed into *Escherichia coli* strain MM294 (American Type Culture Collection *E. Coli.* 33625). All mutants are confirmed by DNA sequencing and the isolated DNA is transformed into the *Bacillus subtilis* expression strain BG2036 (Yang, M. Y., E. Ferrari and D. J. Henner, (1984), "Cloning of the Neutral Protease Gene of *Bacillus subtillis* and the Use of the Cloned Gene to Create an *In Vitro*-derived Deletion Mutation", JOURNAL OF BACTERIOLOGY, Vol. 160, pp. 15-21). For some of the mutants a modified pSS-5 with a frameshift-stop codon mutation at amino acid 217 is used to produce the uracil template. Oligonucleotides are designed to restore the proper reading frame at position 217 and also encoded for random substitutions at positions 199, 200, 201, 202, 203, 204, 205, 206, 207, 208, 209, 210, 211, 212, 213, 214, 215, 216, 217, 218, 219 and 220 (equimolar and/or variable mixtures of all four nucleotides for all three bases at these codons). Mutations that correct for the frameshift-stop and produce a functional

enzyme are identified by their ability to digest casein. The random substitutions are determined by DNA sequencing.

Example 2

Fermentation

The *Bacillus subtilis* cells (BE2036) containing a subtilisin mutant of interest are grown to mid-log phase in a one liter culture of LB-glucose broth and inoculated into a Biostat ED fermenter (B. Braun Biotech, Inc., Allentown, Pennsylvania) in a total volume of 10 liters. The fermentation media contains Yeast Extract, starch, antifoam, buffers and trace minerals (see FERMENTATION: A PRACTICAL APPROACH, Ed. B. McNeil and L. M. Harvey, 1990). The broth is kept at a constant pH of 7.0 during the fermentation run. Chloramphenical is added for antibiotic selection of mutagenized plasmid. The cells are grown overnight at 37°C to an A_{600} of about 60 and harvested.

Example 3

Purification

The fermentation broth is taken through the following steps to obtain pure enzyme. The broth is cleared of *Bacillus subtilis* cells by centrifugation, and clarified by removing fine particulates with a 100K cutoff membrane. This is followed by concentration on a 10K cutoff membrane, and flow dialysis to reduce the ionic strength and adjust the pH to 5.5 using 0.025M MES buffer (2-(*N*-morpholino)ethanesulfonic acid). The enzyme is further purified by loading it onto either a cation exchange chromatography column or an affinity adsorption chromatography column and eluting it from the column with a NaCl or a propylene glycol gradient (*see* Scopes, R. K., PROTEIN PURIFICATION PRINCIPLES AND PRACTICE, Springer-Verlag, New York (1984), incorporated herein by reference).

The *p*NA assay (DelMar, E.G., C. Largman, J.W. Brodrick and M.C. Geokas, ANAL. BIOCHEM., Vol. 99, pp. 316-320, (1979), incorporated herein by reference) is used to determine the active enzyme concentration for fractions collected during gradient elution. This assay measures the rate at which *p*-nitroaniline is released as the enzyme hydrolyzes the soluble synthetic substrate, succinyl-alanine-alanine-proline-phenylalanine-*p*-nitroanilide (sAAPF-*p*NA). The rate of production of yellow color from the hydrolysis reaction is measured at 410 nm on a spectrophotometer and is proportional to the active enzyme concentration. In addition, absorbance measurements at 280 nm are used to determine the total protein

concentration. The active enzyme/total-protein ratio gives the enzyme purity, and is used to identify fractions to be pooled for the stock solution.

To avoid autolysis of the enzyme during storage, an equal weight of propylene glycol is added to the pooled fractions obtained from the chromatography column. Upon completion of the purification procedure the purity of the stock enzyme solution is checked with SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) and the absolute enzyme concentration is determined via an active site titration method using trypsin inhibitor type II-T: turkey egg white purchased from Sigma Chemical Company (St. Louis, Missouri). The measured conversion factors will show which changes made in the enzyme molecule at the various positions result in the enzyme variant having increased activity over the wild-type, against the soluble substrate *p*NA.

In preparation for use, the enzyme stock solution is eluted through a Sephadex-G25 (Pharmacia, Piscataway, New Jersey) size exclusion column to remove the propylene glycol and exchange the buffer. The MES buffer in the enzyme stock solution is exchanged for 0.1 M Tris buffer (Tris(hydroxymethyl-aminomethane) containing 0.01M $CaCl_2$ and pH adjusted to 8.6 with HCl. All experiments are carried out at pH 8.6 in Tris buffer thermostated at 25°C.

E. Characterization of enzyme variants

Example 4

Model Surface Preparation

Aminopropyl controlled pore glass (CPG) purchased from CPG Inc. (Fairfield, New Jersey) is used as a support for covalently attaching the sAAPF-*p*NA substrate purchased from Bachem, Inc. (Torrence, California). The reaction is carried out in dimethyl sulfoxide and (1-ethyl-3-[3-(dimethylamino)propyl] carbodiimide hydrochloride) (EDC) is used as a coupling agent. Upon completion (monitored by *p*NA assay), the excess solvent is removed, and the CPG:sAAPF-*p*NA is rinsed with dimethyl sulfoxide (DMSO) and doubly-distilled water. This is followed by oven drying with a N_2 purge at about 70°C. The reaction scheme and preparation of the immobilized substrate are conducted as described by Brode, P.F. III, and D.S. Rauch, "Subtilisin BPN': Activity on an Immobilized Substrate," LANGMUIR, Vol. 8, p. 1325-1329, (1992), incorporated herein by reference.

The CPG surface will have 62,000 ± 7,000 *p*NA molecules/μm^2. The surface area will remain unchanged from the value of 50.0m^2/g reported by

CPG Inc. for the CPG as received. This suggests that the procedure used to add sAAPF-*p*NA to CPG does not damage the porous structure (mean diameter is 486 Å).

Example 5

Surface Hydrolysis Assay

Using CPG:sAAPF-*p*NA, adsorption of an enzyme variant and hydrolysis of a CPG-bound peptide can be measured in a single experiment. A small volume of enzyme variant stock solution is added to a flask containing Tris buffer and CPG:sAAPF-*p*NA which has been degassed. The flask is shaken on a wrist-action shaker for a period of 90 minutes during which the shaker is stopped at various time intervals (for example, every 2 minutes during the early stages of adsorption hydrolysis - e.g., the first 20 minutes - and every 10 minutes towards the end of the experiment). The CPG:sAAPF-*p*NA is allowed to settle and the solution is sampled. Both the experimental procedure and the calculation of the adsorption and hydrolysis are conducted as described by Brode *et al.*, 1992, above.

All enzymes are monitored for stability against autolysis and should show no appreciable autolytic loss over the time course of this experiment. Therefore, enzyme adsorption can be determined by measuring solution depletion. The difference between the initial enzyme variant concentration and the concentration measured at each individual time point gives the amount of enzyme variant adsorbed. The amount of *p*NA hydrolyzed from the surface is measured by taking an absorbance reading on an aliquot of the sample at 410 nm. The total amount of *p*NA hydrolyzed is calculated by adding the amount sampled and the amount remaining in the flask. This value is corrected by subtracting the amount of *p*NA that is hydrolyzed by Tris buffer at pH 8.6 when no enzyme is present. This base-hydrolysis ranges from 7-29% of the total hydrolysis depending on the efficiency of the enzyme.

Example 6

Soluble Substrate Kinetic Analysis

The rates of hydrolysis of the soluble substrate sAAPF-*p*NA are monitored by measuring the adsorbance increase as a function of time at 410 nm on a DU-70 spectrophotometer. The enzyme concentration is held constant and is prepared to be in the range of 6-10 nanomolar while the substrate concentration is varied from 90-700 μM sAAPF-*p*NA for each kinetic determination. An adsorbance data point is taken each second over

a period of 900 seconds and the data are transferred to a Lotus™ spreadsheet (Lotus Development Corporation, Cambridge, Massachusetts). Analysis for kinetic parameters is conducted by the standard Lineweaver Burk analysis in which the data in the initial part of the run (generally the first minute) are fit to a linear regression curve to give v_o. The v_o and s_o data are plotted in the standard inverse fashion to give K_M and k_{cat}.

F. Example BPN' variants

BPN' variants of the present invention which have decreased adsorption to and increased hydrolysis of surface bound substrates are exemplified in Table 2, below. In describing the specific mutations, the original amino acid occurring in wild-type is given first, the position number second, and the substituted amino acid third.

TABLE 2

Example BPN' Variants

--Single Mutation--

Ala216Glu
Ala216Asp
Ala216Gly
Val203Glu

--Double Mutation--

Ile205Leu + Ala216Glu
Ile205Leu + Ala216Asp
Pro210Ala + Gly215Thr
Tyr214Phe + Tyr217Asn
Gln206Glu + Ala216Glu
Ala216Glu + Try217Leu
Gln206Glu + Tyr217Leu

--Triple Mutation--

Gln206Glu + Ala216Glu + Tyr217Leu
Gln206Pro + Gly211Ala + Ala216Glu

--Quadruple Mutation--

Val203Glu + Gln206Glu + Ala216Glu + Tyr217Leu
Val203Glu + Pro210Ala + Ala216Glu + Tyr217Leu

--Quintuple Mutation--

Val203Glu + Gln206Glu + Gly215Thr + Ala216Glu + Tyr217Leu
Val203Glu + Pro210Ala + Gly215Thr + Ala216Glu + Tyr217Leu

II. Fabric Cleaning Composition Materials

The fabric cleaning compositions of the present invention also comprise, in addition to the BPN' variants described hereinbefore, one or more cleaning composition materials compatible with the protease enzyme.

The term "cleaning composition material", as used herein, means any liquid, solid or gaseous material selected for the particular type of cleaning composition desired and the form of the product (e.g., liquid; granule; bar), which materials are also compatible with the BPN' variant used in the composition. The specific selection of cleaning composition materials are readily made by considering the fabric to be cleaned, and the desired form of the composition for the cleaning condition during use (e.g., through the wash detergent use). The term "compatible", as used herein, means the cleaning composition materials do not reduce the proteolytic activity of the BPN' variant to such an extent that the protease is not effective as desired during normal use situations. Specific cleaning composition materials are exemplified in detail hereinafter.

As used herein, "fabric cleaning composition" refers to all forms for detergent compositions for cleaning fabrics, including but not limited to, granular, liquid and bar forms. Preferred fabric cleaning compositions are those in the liquid form.

As used herein, "effective amount of enzyme variant" refers to the quantity of enzyme variant necessary to achieve the enzymatic activity necessary in the specific cleaning composition. Such effective amounts are readily ascertained by one of ordinary skill in the art and is based on many factors, such as the particular enzyme variant used, the cleaning application, the specific composition of the cleaning composition, and whether a liquid or dry (e.g., granular) composition is required, and the like. Preferably the cleaning compositions of the present invention comprise from about 0.0001% to about 10% of one or more enzyme variants, more preferably from about 0.001% to about 1%, more preferably still from about 0.01% to about 0.1%. Several examples of various cleaning compositions of the present invention are discussed in further detail below. All parts, percentages and ratios used herein are by weight unless otherwise specified.

The enzyme variants of the present invention can be used with various conventional ingredients to provide fully-formulated fabric laundering compositions. Such compositions can be in the form of liquids, granules and the like. Such compositions can be formulated as modern "concentrated" detergents which contain as much as 30%-60% by weight of surfactants.

The fabric cleaning compositions herein can optionally, and preferably, contain various anionic, nonionic, zwitterionic, etc., surfactants.

Such surfactants are typically present at levels of from about 5% to about 35% of the compositions.

Nonlimiting examples of surfactants useful herein include the conventional C_{11}-C_{18} alkyl benzene sulfonates and primary and random alkyl sulfates, the C_{10}-C_{18} secondary (2,3) alkyl sulfates of the formulas $CH_3(CH_2)x(CHOSO_3^-M^+)CH_3$ and $CH_3(CH_2)y(CHOSO_3^-M^+)$ CH_2CH_3 wherein x and (y+1) are integers of at least about 7, preferably at least about 9, and M is a water-solubilizing cation, especially sodium, the C_{10}-C_{18} alkyl alkoxy sulfates (especially EO 1-5 ethoxy sulfates), C_{10}-C_{18} alkyl alkoxy carboxylates (especially the EO 1-5 ethoxycarboxylates), the C_{10}-C_{18} alkyl polyglycosides, and their corresponding sulfated polyglycosides, C_{12}-C_{18} alpha-sulfonated fatty acid esters, C_{12}-C_{18} alkyl and alkyl phenol alkoxylates (especially ethoxylates and mixed ethoxy/propoxy), C_{12}-C_{18} betaines and sulfobetaines ("sultaines"), C_{10}-C_{18} amine oxides, and the like. The alkyl alkoxy sulfates (AES) and alkyl alkoxy carboxylates (AEC) are preferred herein. (Use of such surfactants in combination with the aforesaid amine oxide and/or betaine or sultaine surfactants is also preferred, depending on the desires of the formulator.) Other conventional useful surfactants are listed in standard texts. Particularly useful surfactants include the C_{10}-C_{18} N-methyl glucamides disclosed in US Patent 5, 194,639, Connor et al., issued March 16, 1993, incorporated herein by reference.

A wide variety of other ingredients useful in fabric cleaning compositions can be included in the compositions herein, including other active ingredients, carriers, hydrotropes, processing aids, dyes or pigments, solvents for liquid formulations, etc. If an additional increment of sudsing is desired, suds boosters such as the C_{10}-C_{16} alkolamides can be incorporated into the compositions, typically at about 1% to about 10% levels. The C_{10}-C_{14} monoethanol and diethanol amides illustrate a typical class of such suds boosters. Use of such suds boosters with high sudsing adjunct surfactants such as the amine oxides, betaines and sultaines noted above is also advantageous. If desired, soluble magnesium salts such as $MgCl_2$, $MgSO_4$, and the like, can be added at levels of, typically, from about 0.1% to about 2%, to provide additionally sudsing.

The liquid fabric cleaning compositions herein can contain water and other solvents as carriers. Low molecular weight primary or secondary alcohols exemplified by methanol, ethanol, propanol, and isopropanol are suitable. Monohydric alcohols are preferred for solubilizing surfactants, but

polyols such as those containing from about 2 to about 6 carbon atoms and from about 2 to about 6 hydroxy groups (e.g., 1,3-propanediol, ethylene glycol, glycerine, and 1,2-propanediol) can also be used. The compositions may contain from about 5% to about 90%, typically from about 10% to about 50% of such carriers.

The fabric cleaning compositions herein will preferably be formulated such that during use in aqueous cleaning operations, the wash water will have a pH between about 6.8 and about 11.0. Finished products thus are typically formulated at this range. Techniques for controlling pH at recommended usage levels include the use of buffers, alkalis, acids, etc., and are well known to those skilled in the art.

When formulating the fabric cleaning compositions of the present invention, the formulator may wish to employ various builders at levels from about 5% to about 50% by weight. Typical builders include the 1-10 micron zeolites, polycarboxylates such as citrate and oxydisuccinates, layered silicates, phosphates, and the like. Other conventional builders are listed in standard formularies.

Likewise, the formulator may wish to employ various additional enzymes, such as cellulases, lipases, amylases and proteases in such compositions, typically at levels of from about 0.001% to about 1% by weight. Various fabric care enzymes are well-known in the laundry detergent art.

Various bleaching compounds, such as the percarbonates, perborates and the like, can be used in such compositions, typically at levels from about 1% to about 15% by weight. If desired, such compositions can also contain bleach activators such as tetraacetyl ethylenediamine, nonanoyloxybenzene sulfonate, and the like, which are also known in the art. Usage levels typically range from about 1% to about 10% by weight.

Various soil release agents, especially of the anionic oligoester type, various chelating agents, especially the aminophosphonates and ethylenediaminedisuccinates, various clay soil removal agents, especially ethoxylated tetraethylene pentamine, various dispersing agents, especially polyacrylates and polyasparatates, various brighteners, especially anionic brighteners, various suds suppressors, especially silicones and secondary alcohols, various fabric softeners, especially smectite clays, and the like can all be used in such compositions at levels ranging from about 1% to about 35% by weight. Standard formularies and published patents contain multiple, detailed descriptions of such conventional materials.

Enzyme stabilizers may also be used in the cleaning compositions of the present invention. Such enzyme stabilizers include propylene glycol (preferably from about 1% to about 10%), sodium formate (preferably from about 0.1% to about 1%) and calcium formate (preferably from about 0.1% to about 1%).

a. Granular fabric cleaning compositions

The granular fabric cleaning compositions of the present invention contain an effective amount of one or more enzyme variants of the present invention, preferably from about 0.001% to about 10%, more preferably from about 0.005% to about 5%, more preferably from about 0.01% to about 1% by weight of active enzyme of the composition. In addition to one or more enzyme variants, the granular fabric cleaning compositions typically comprise at least one surfactant, one or more builders, and, in some cases, a bleaching agent.

The granular fabric cleaning composition embodiment of the present invention is illustrated by the following examples.

Examples 7-10

Granular Fabric Cleaning Composition

	Example No.			
Component	7	8	9	10
Ala216Glu	0.10	0.20	0.03	0.05
Gln206Glu + Tyr217Leu	-	-	0.02	0.05
C_{13} linear alkyl benzene sulfonate	22.00	22.00	22.00	22.00
Phosphate (as sodium tripolyphosphates)	23.00	23.00	23.00	23.00
Sodium carbonate	23.00	23.00	23.00	23.00
Sodium silicate	14.00	14.00	14.00	14.00
Zeolite	8.20	8.20	8.20	8.20
Chelant (diethylaenetriamine-pentaacetic acid)	0.40	0.40	0.40	0.40
Sodium sulfate	5.50	5.50	5.50	5.50
Water	balance to 100%			

In Examples 7-8, the BPN' variants recited in Table 2, among others, are substituted for Ala216Glu, with substantially similar results.

In Examples 9-10, any combination of the BPN' variants recited in Table 2, among others, are substituted for Ala216Glu and Gln206Glu + Tyr217Leu, with substantially similar results.

Examples 11-14

Granular Fabric Cleaning Composition

Component	Example No. 11	12	13	14
Gln206Glu + Ala216Glu + Tyr217Leu	0.10	0.20	0.03	0.05
Pro210Ala + Gly215Thr	-	-	0.02	0.05
C_{12} alkyl benzene sulfonate	12.00	12.00	12.00	12.00
Zeolite A (1-10 micrometer)	26.00	26.00	26.00	26.00
2-butyl octanoic acid	4.00	4.00	4.00	4.00
C_{12}-C_{14} secondary (2,3) alkyl sulfate, Na salt	5.00	5.00	5.00	5.00
Sodium citrate	5.00	5.00	5.00	5.00
Optical brightener	0.10	0.10	0.10	0.10
Sodium sulfate	17.00	17.00	17.00	17.00
Water and minors	balance to 100%			

In Examples 11-12, the BPN' variants recited in Table 2, among others, are substituted for Gln206Glu + Ala216Glu + Tyr217Leu, with substantially similar results.

In Examples 13-14, any combination of the BPN' variants recited in Table 2, among others, are substituted for Gln206Glu + Ala216Glu + Tyr217Leu and Pro210Ala + Gly215Thr, with substantially similar results.

Examples 15 and 16

Granular Fabric Cleaning Compositions

Components	Example No. 15	16
Linear alkyl benzene sulphonate	11.4	10.70
Tallow alkyl sulphate	1.80	2.40
C_{14-15} alkyl sulphate	3.00	3.10
C_{14-15} alcohol 7 times ethoxylated	4.00	4.00
Tallow alcohol 11 times ethoxylated	1.80	1.80
Dispersant	0.07	0.1
Silicone fluid	0.80	0.80
Trisodium citrate	14.00	15.00
Citric acid	3.00	2.50
Zeolite	32.50	32.10
Maleic acid acrylic acid copolymer	5.00	5.00
Diethylene triamine penta methylene phosphonic acid	1.00	0.20

Ala216Glu + Tyr217Leu	0.30	0.30
Lipase	0.36	0.40
Amylase	0.30	0.30
Sodium silicate	2.00	2.50
Sodium sulphate	3.50	5.20
Polyvinyl pyrrolidone	0.30	0.50
Perborate	0.5	1
Phenol sulphonate	0.1	0.2
Peroxidase	0.1	0.1
Minors	Up to 100	Up to 100

Examples 17 and 18

Granular Fabric Cleaning Compositions

	Example No.	
Components	17	18
Sodium linear C_{12} alkyl benzene-sulfonate	6.5	8.0
Sodium sulfate	15.0	18.0
Zeolite A	26.0	22.0
Sodium nitrilotriacetate	5.0	5.0
Polyvinyl pyrrolidone	0.5	0.7
Tetraacetylethylene diamine	3.0	3.0
Boric acid	4.0	-
Perborate	0.5	1
Phenol sulphonate	0.1	0.2
Ile205Leu + Ala216Glu	0.4	0.4
Fillers (e.g., silicates; carbonates; perfumes; water)	Up to 100	Up to 100

Example 19

Compact Granular Fabric Cleaning Composition

Components	Weight %
Alkyl Sulphate	8.0
Alkyl Ethoxy Sulphate	2.0
Mixture of C_{25} and C_{45} alcohol 3 and 7 times ethoxylated	6.0
Polyhydroxy fatty acid amide	2.5
Zeolite	17.0
Layered silicate/citrate	16.0
Carbonate	7.0
Maleic acid acrylic acid copolymer	5.0
Soil release polymer	0.4

Carboxymethyl cellulose	0.4
Poly (4-vinylpyridine) -N-oxide	0.1
Copolymer of vinylimidazole and vinylpyrrolidone	0.1
PEG2000	0.2
Val203Glu + Gln206Glu + Ala216Glu + Tyr217Leu	0.5
Lipase	0.2
Cellulase	0.2
Tetracetylethylene diamine	6.0
Percarbonate	22.0
Ethylene diamine disuccinic acid	0.3
Suds suppressor	3.5
Disodium-4,4'-bis (2-morpholino -4-anilino-s-triazin-6-ylamino) stilbene-2,2'-disulphonate	0.25
Disodium-4,4'-bis (2-sulfostyril) biphenyl	0.05
Water, Perfume and Minors	Up to 100

Example 20

Granular Fabric Cleaning Composition

Component	Weight %
Linear alkyl benzene sulphonate	7.6
C_{16}-C_{18} alkyl sulfate	1.3
C_{14-15} alcohol 7 times ethoxylated	4.0
Coco-alkyl-dimethyl hydroxyethyl ammonium chloride	1.4
Dispersant	0.07
Silicone fluid	0.8
Trisodium citrate	5.0
Zeolite 4A	15.0
Maleic acid acrylic acid copolymer	4.0
Diethylene triamine penta methylene phosphonic acid	0.4
Perborate	15.0
Tetraacetylethylene diamine	5.0
Smectite clay	10.0
Poly (oxy ethylene) (MW 300,000)	0.3
Tyr214Phe + Tyr217Asn	0.4
Lipase	0.2
Amylase	0.3
Cellulase	0.2
Sodium silicate	3.0
Sodium carbonate	10.0

Carboxymethyl cellulose	0.2
Brighteners	0.2
Water, perfume and minors	Up to 100

Example 21

Granular Fabric Cleaning Composition

Component	Weight %
Linear alkyl benzene sulfonate	6.92
Tallow alkyl sulfate	2.05
C_{14-15} alcohol 7 times ethoxylated	4.4
C_{12-15} alkyl ethoxy sulfate - 3 times ethoxylated	0.16
Zeolite	20.2
Citrate	5.5
Carbonate	15.4
Silicate	3.0
Maleic acid acrylic acid copolymer	4.0
Carboxymethyl cellulase	0.31
Soil release polymer	0.30
Val203Glu + Pro210Ala + Gly215Thr + Ala216Glu + Tyr217Leu	0.2
Lipase	0.36
Cellulase	0.13
Perborate tetrahydrate	11.64
Perborate monohydrate	8.7
Tetraacetylethylene diamine	5.0
Diethylene tramine penta methyl phosphonic acid	0.38
Magnesium sulfate	0.40
Brightener	0.19
Perfume, silicone, suds suppressors	0.85
Minors	Up to 100

b. Liquid fabric cleaning compositions

Liquid fabric cleaning compositions of the present invention comprise an effective amount of one or more enzyme variants of the present invention, preferably from about 0.005% to about 5%, more preferably from about 0.01% to about 1%, by weight of active enzyme of the composition. Such liquid fabric cleaning compositions typically additionally comprise an anionic surfactant, a fatty acid, a water-soluble detergency builder and water.

The liquid fabric cleaning composition embodiment of the present invention is illustrated by the following examples.

Examples 22-26

Liquid Fabric Cleaning Compositions

	Example No.				
Component	22	23	24	25	26
Gln206Glu + Ala216Glu + Tyr217Leu	0.05	0.03	0.30	0.03	0.10
Pro210Ala + Gly215Thr	–	–	–	0.01	0.20
C_{12}- C_{14} alkyl sulfate, Na	20.00	20.00	20.00	20.00	20.00
2-butyl octanoic acid	5.00	5.00	5.00	5.00	5.00
Sodium citrate	1.00	1.00	1.00	1.00	1.00
C_{10} alcohol ethoxylate (3)	13.00	13.00	13.00	13.00	13.00
Monethanolamine	2.50	2.50	2.50	2.50	2.50
Water/propylene glycol/ethanol (100:1:1)	balance to 100%				

In Examples 22-24 the BPN' variants recited in Table 2, among others, are substituted for Gln206Glu + Ala216Glu + Tyr217Leu, with substantially similar results.

In Examples 25-26, any combination of the BPN' variants recited in Table 2, among others, are substituted for Gln206Glu + Ala216Glu + Tyr217Leu and Pro210Ala + Gly215Thr, with substantially similar results.

Examples 27-28

Liquid Fabric Cleaning Compositions

	Example No.	
Component	27	28
C_{12-14} alkenyl succinic acid	3.0	8.0
Citric acid monohydrate	10.0	15.0
Sodium C_{12-15} alkyl sulphate	8.0	8.0
Sodium sulfate of C_{12-15} alcohol 2 times ethoxylated	-	3.0
C_{12-15} alcohol 7 times ethoxylated	-	8.0
C_{12-15} alcohol 5 times ethoxylated	8.0	-
Diethylene triamine penta (methylene phosphonic acid)	0.2	-
Oleic acid	1.8	-
Ethanol	4.0	4.0
Propanediol	2.0	2.0
Ala216Glu +Tyr217Leu	0.2	0.2
Polyvinyl pyrrolidone	1.0	2.0

Suds suppressor	0.15	0.15
NaOH	up to pH 7.5	
Perborate	0.5	1
Phenol sulphonate	0.1	0.2
Peroxidase	0.4	0.1
Waters and minors	up to 100 parts	

In each of Examples 27 and 28 herein, the BPN' variants recited in Table 2, among others, are substituted for Ala216Glu +Tyr217Leu, with substantially similar results.

Examples 29-31

Liquid Fabric Cleaning Compositions

	Example No.		
Component	29	30	31
Citric Acid	7.10	3.00	3.00
Fatty Acid	-	2.00	2.00
Ethanol	1.93	3.20	3.20
Boric Acid	2.22	3.50	3.50
Monoethanolamine	0.71	1.09	1.09
1,2 Propanediol	7.89	8.00	8.00
NaCumene Sulfonate	1.80	3.00	3.00
NaFormate	0.08	0.08	0.08
NaOH	6.70	3.80	3.80
Silicon anti-foam agent	1.16	1.18	1.18
Ala216Glu	0.0145	-	-
Ala216Glu + Tyr217Leu	-	0.0145	-
Gln206Glu + Ala216Glu + Tyr217Leu	-	-	0.0145
Lipase	.200	.200	.200
Cellulase	-	7.50	7.50
Soil release polymer	0.29	0.15	0.15
Anti-foaming agents	0.06	0.085	0.085
Brightener 36	0.095	-	-
Brightener 3	-	0.05	0.05
C_{12} alkyl benzenesulfonic acid	9.86	-	-
C_{12-15} alkyl polyethoxylate (2.5) sulfate	13.80	18.00	18.00
C_{12} glucose amide	-	5.00	5.00
C_{12-13} alkyl polyethoxylate (9)	2.00	2.00	2.00
Water, perfume and minors	balance to 100%		

c. Bar fabric cleaning compositions

Bar fabric cleaning compositions of the present invention suitable for hand-washing soiled fabrics contain an effective amount of one or more enzyme variants of the present invention, preferably from about 0.001% to about 10%, more preferably from about 0.01% to about 1% by weight of the composition.

The bar fabric cleaning composition embodiment of the present invention is illustrated by the following examples.

Examples 33-36

Bar Fabric Cleaning Compositions

	Example No.			
Component	33	34	35	36
Val203Glu	0.3	-	0.1	0.02
Tyr214Phe + Tyr217Asn	-	0.3	0.4	0.03
C_{12}-C_{16} alkyl sulfate, Na	20.0	20.0	20.0	20.00
C_{12}-C_{14} N-methyl glucamide	5.0	5.0	5.0	5.00
C_{11}-C_{13} alkyl benzene sulfonate, Na	10.0	10.0	10.0	10.00
Sodium carbonate	25.0	25.0	25.0	25.00
Sodium pyrophosphate	7.0	7.0	7.0	7.00
Sodium tripolyphosphate	7.0	7.0	7.0	7.00
Zeolite A (0.1-.10μ)	5.0	5.0	5.0	5.00
Carboxymethylcellulose	0.2	0.2	0.2	0.20
Polyacrylate (MW 1400)	0.2	0.2	0.2	0.20
Coconut monethanolamide	5.0	5.0	5.0	5.00
Brightener, perfume	0.2	0.2	0.2	0.20
$CaSO_4$	1.0	1.0	1.0	1.00
$MgSO_4$	1.0	1.0	1.0	1.00
Water	4.0	4.0	4.0	4.00
Filler*	balance to 100%			

*Can be selected from convenient materials such as $CaCO_3$, talc, clay, silicates, and the like.

In Example 33, the BPN' variants recited in Table 2, among others, are substituted for Val203Glu, with substantially similar results.

In Example 34, the BPN' variants recited in Table 2, among others, are substituted for Tyr214Phe + Tyr217Asn, with substantially similar results.

In Examples 35-36, any combination of the BPN' variants recited in Table 2, among others, are substituted for Val203Glu and Tyr214Phe + Tyr217Asn, with substantially similar results.

While particular embodiments of the subject invention have been disclosed, it will be obvious to those skilled in the art that various changes and modifications of the subject invention can be made without departing from the spirit and scope of the invention. It is intended to cover, in the appended claims, all such modifications that are within the scope of the invention.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: BRODE, PHILIP F.
SARNETT, BOBBY L.
RUBINGH, DONN N.
GHOSH, CHANCHAL K.

(ii) TITLE OF INVENTION: SERINE PROTEASE CONTAINING FABRIC CLEANING COMPOSITIONS

(iii) NUMBER OF SEQUENCES: 1

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: THE PROCTER & GAMBLE COMPANY
(B) STREET: 11810 EAST MIAMI RIVER ROAD
(C) CITY: ROSS
(D) STATE: OH
(E) COUNTRY: USA
(F) ZIP: 45061

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
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(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: ROOF, CARL J.
(B) REGISTRATION NUMBER: 37,708
(C) REFERENCE/DOCKET NO. 5232

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 513-627-0081
(B) TELEFAX: 513-627-0260

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 275 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```
Ala Gln Ser Val Pro Tyr Gly Val Ser Gln Ile Lys Ala Pro Ala Leu
1               5                  10                  15

His Ser Gln Gly Tyr Thr Gly Ser Asn Val Lys Val Ala Val Ile Asp
            20                  25                  30

Ser Gly Ile Asp Ser Ser His Pro Asp Leu Lys Val Ala Gly Gly Ala
        35                  40                  45
```

Ser Met Val Pro Ser Glu Thr Asn Pro Phe Gln Asp Asn Asn Ser His
50 55 60

Gly Thr His Val Ala Gly Thr Val Ala Ala Leu Asn Asn Ser Ile Gly
65 70 75 80

Val Leu Gly Val Ala Pro Ser Ala Ser Leu Tyr Ala Val Lys Val Leu
85 90 95

Gly Ala Asp Gly Ser Gly Gln Tyr Ser Trp Ile Ile Asn Gly Ile Glu
100 105 110

Trp Ala Ile Ala Asn Asn Met Asp Val Ile Asn Met Ser Leu Gly Gly
115 120 125

Pro Ser Gly Ser Ala Ala Leu Lys Ala Ala Val Asp Lys Ala Val Ala
130 135 140

Ser Gly Val Val Val Val Ala Ala Ala Gly Asn Glu Gly Thr Ser Gly
145 150 155 160

Ser Ser Ser Thr Val Gly Tyr Pro Gly Lys Tyr Pro Ser Val Ile Ala
165 170 175

Val Gly Ala Val Asp Ser Ser Asn Gln Arg Ala Ser Phe Ser Ser Val
180 185 190

Gly Pro Glu Leu Asp Val Met Ala Pro Gly Val Ser Ile Gln Ser Thr
195 200 205

Leu Pro Gly Asn Lys Tyr Gly Ala Tyr Asn Gly Thr Ser Met Ala Ser
210 215 220

Pro His Val Ala Gly Ala Ala Ala Leu Ile Leu Ser Lys His Pro Asn
225 230 235 240

Trp Thr Asn Thr Gln Val Arg Ser Ser Leu Glu Asn Thr Thr Thr Lys
245 250 255

Leu Gly Asp Ser Phe Tyr Tyr Gly Lys Gly Leu Ile Asn Val Gln Ala
260 265 270

Ala Ala Gln
275

Claims:

1. A fabric cleaning composition comprising:

(a) an effective amount of a BPN' variant comprising wild-type amino acid sequence wherein the wild-type amino acid sequence at one or more of positions 199, 200, 201, 202, 203, 204, 205, 206, 207, 208, 209, 210, 211, 212, 213, 214, 215, 216, 218, 219 or 220 is substituted, characterized in that

i. when a substitution occurs at position 199, the substituting amino acid for position 199 is Cys, Ala, His, Thr, Pro, Gly, Gln, Asn, Ser, Asp or Glu;

ii. when a substitution occurs at position 200, the substituting amino acid for position 200 is His, Thr, Pro, Gly, Gln, Asn, Ser, Asp or Glu;

iii. when a substitution occurs at position 201, the substituting amino acid for position 201 is Gly, Gln, Asn, Ser, Asp or Glu;

iv. when a substitution occurs at position 202, the substituting amino acid for position 202 is Pro, Gln, Asn, Ser, Asp or Glu;

v. when a substitution occurs at position 203, the substituting amino acid for position 203 is Met, Cys, His, Pro, Gly, Gln, Asn, Ser, Asp or Glu;

vi. when a substitution occurs at position 204, the substituting amino acid for position 204 is Glu;

vii. when a substitution occurs at position 205, the substituting amino acid for position 205 is Leu, Met, Cys, Ala, His, Thr, Pro, Gly, Gln, Asn, Ser, Asp or Glu;

viii. when a substitution occurs at position 206, the substituting amino acid for position 206 is Pro, Asn or Ser;

ix. when a substitution occurs at position 207, the substituting amino acid for position 207 is Asp or Glu;

x. when a substitution occurs at position 208, the substituting amino acid for position 208 is Pro, Gly, Gln, Asn, Ser, Asp or Glu;

xi. when a substitution occurs at position 209, the substituting amino acid for position 209 is Ile, Val, Met, Cys, Ala, His, Thr, Pro, Gly, Gln, Asn, Ser, Asp or Glu;

xii. when a substitution occurs at position 210, the substituting amino acid for position 210 is Gly, Gln, Asn, Ser, Asp or Glu;

xiii. when a substitution occurs at position 211, the substituting amino acid for position 211 is Ala, Pro, Gln, Asn, Ser, Asp or Glu;

xiv. when a substitution occurs at position 212, the substituting amino acid for position 212 is Gln, Ser, Asp or Glu;

xv. when a substitution occurs at position 213, the substituting amino acid for position 213 is Trp, Phe, Tyr, Leu, Ile, Val, Met, Cys, Ala, His, Pro, Gly, Gln, Asn, Ser, Asp or Glu;

xvi. when a substitution occurs at position 214, the substituting amino acid for position 214 is Phe, Leu, Ile, Val, Met, Cys, Ala, His, Pro, Gly, Gln, Asn, Asp or Glu;

xvii. when a substitution occurs at position 215, the substituting amino acid for position 215 is Thr, Pro, Gln, Asn, Ser, Asp or Glu;

xviii. when a substitution occurs at position 216, the substituting amino acid for position 216 is His, Thr, Pro, Gly, Gln, Asn, Ser, Asp or Glu;

xix. when a substitution occurs at position 218, the substituting amino acid for position 218 is Glu;

xx. when a substitution occurs at position 219, the substituting amino acid for position 219 is Pro, Gln, Asn, Ser, Asp; or Glu; and

xxi. when a substitution occurs at position 220, the substituting amino acid for position 220 is Pro, Gly, Gln, Asn, Asp or Glu;

characterized in that the BPN' variant has decreased adsorption to, and increased hydrolysis of, an insoluble substrate as compared to wild-type subtilisin BPN'; and

(b) one or more cleaning composition materials compatible with the BPN' variant.

2. The fabric cleaning composition of Claim 1, characterized in that

a. when a substitution occurs at position 206, the substituting amino acid for position 206 is Asn or Ser;

b. when a substitution occurs at position 211, the substituting amino acid for position 211 is Pro, Gln, Asn, Ser, Asp or Glu;

c. when a substitution occurs at position 214, the substituting amino acid for position 214 is Leu, Ile, Val, Met, Cys, Ala, His, Pro, Gly, Gln, Asn, Asp or Glu; and

d. when a substitution occurs at position 215, the substituting amino acid for position 215 is Pro, Gln, Asn, Ser, Asp or Glu.

3. The fabric cleaning composition of Claim 2, characterized in that when position 216 is substituted, Gly is substituted for Ala at position 216.

4. The fabric cleaning composition of Claim 2, characterized in that when a substitution occurs at one or more of positions 199, 200, 201, 202, 203, 205, 207, 208, 209, 210, 211, 212, 213, 214, 215, 216, 219 or 220, the substituting amino acid for any of positions 199, 200, 201, 202, 203, 205, 207, 208, 209, 210, 211, 212, 213, 214, 215, 216, 219 or 220 is Asp or Glu; and when a substitution occurs at one or both of positions 204 or 208, the substituting amino acid for positions 204 or 218 is Glu; and wherein a substitution preferably occurs at one or more of positions of 199, 200, 201, 202, 205, 207, 208, 209, 210, 211, 212 or 215, more preferably at one or more of positions 200, 201, 202, 205 or 207.

5. The fabric cleaning composition of Claim 1 having a single amino acid substitution characterized in that the substitution is:

a. Glu for Ala at position 216;

b. Asp for Ala at position 216; or

c. Glu for Val at position 203.

6. A fabric cleaning composition comprising:

(a) a BPN' variant comprising wild-type amino acid sequence characterized in that the wild-type amino acid sequence at two or more of positions 199, 200, 201, 202, 203, 204, 205, 206, 207, 208, 209, 210, 211, 212, 213, 214, 215, 216, 217, 218, 219 or 220 is substituted, characterized in that

i. when a substitution occurs at position 199, the substituting amino acid for position 199 is Cys, Ala, His, Thr, Pro, Gly, Gln, Asn, Ser, Asp or Glu;

ii. when a substitution occurs at position 200, the substituting amino acid for position 200 is His, Thr, Pro, Gly, Gln, Asn, Ser, Asp or Glu;

iii. when a substitution occurs at position 201, the substituting amino acid for position 201 is Gly, Gln, Asn, Ser, Asp or Glu;

iv. when a substitution occurs at position 202, the substituting amino acid for position 202 is Pro, Gln, Asn, Ser, Asp or Glu;

v. when a substitution occurs at position 203, the substituting amino acid for position 203 is Met, Cys, Ala, His, Thr, Pro, Gly, Gln, Asn, Ser, Asp or Glu;

vi when a substitution occurs at position 204, the substituting amino acid for position 204 is Asp or Glu;

vii. when a substitution occurs at position 205, the substituting amino acid for position 205 is Leu, Val, Met, Cys, Ala, His, Thr, Pro, Gly, Gln, Asn, Ser, Asp or Glu;

viii. when a substitution occurs at position 206, the substituting amino acid for position 206 is Pro, Asn, Ser, Asp, or Glu;

ix. when a substitution occurs at position 207, the substituting amino acid for position 207 is Asp or Glu;

x. when a substitution occurs at position 208, the substituting amino acid for position 208 is Pro, Gly, Gln, Asn or Ser;

xi. when a substitution occurs at position 209, the substituting amino acid for position 209 is Ile, Val,

Met, Cys, Ala, His, Thr, Pro, Gly, Gln, Asn, Ser, Asp or Glu;

xii. when a substitution occurs at position 210, the substituting amino acid for position 210 is Ala, Gly, Gln, Asn, Ser, Asp or Glu;

xiii. when a substitution occurs at position 211, the substituting amino acid for position 211 is Ala, Pro, Gln, Asn, Ser, Asp or Glu;

xiv. when a substitution occurs at position 212, the substituting amino acid for position 212 is Gln, Ser, Asp or Glu;

xv. when a substitution occurs at position 213, the substituting amino acid for position 213 is Trp, Phe, Tyr, Leu, Ile, Val, Met, Cys, Ala, His, Thr, Pro, Gly, Gln, Asn, Ser, Asp or Glu;

xvi. when a substitution occurs at position 214, the substituting amino acid for position 214 is Phe, Leu, Ile, Val, Met, Cys, Ala, His, Thr, Pro, Gly, Gln, Asn or Ser;

xvii. when a substitution occurs at position 215, the substituting amino acid for position 215 is Thr, Pro, Gln, Asn, Ser, Asp or Glu;

xviii. when a substitution occurs at position 216, the substituting amino acid for position 216 is His, Thr, Pro, Gly, Gln, Asn, Ser, Asp or Glu;

xix. when a substitution occurs at position 217, the substituting amino acid for position 217 is Leu, Ile, Val, Met, Cys, Ala, His, Thr, Pro, Gly, Gln, Asn, Ser, Asp or Glu;

xx. when a substitution occurs at position 218, the substituting amino acid for position 218 is Gln, Ser, Asp or Glu;

xxi. when a substitution occurs at position 219, the substituting amino acid for position 219 is Pro, Gln, Asn, Ser, Asp or Glu; and

xxii. when a substitution occurs at position 220, the substituting amino acid for position 220 is Pro, Gly, Gln, Asn, Ser, Asp or Glu;

characterized in that the BPN' variant has decreased adsorption to, and increased hydrolysis of, an insoluble substrate as compared to wild-type subtilisin BPN'; and

(b) one or more cleaning composition materials compatible with the BPN' variant.

7. The fabric cleaning composition of Claim 6, characterized in that the wild-type BPN' is substituted at two positions.

8. The fabric cleaning composition of Claim 7 characterized in that the two substitutions are:

a. Ala for Pro at position 210 and Thr for Gly at position 215;
b. Phe for Tyr at position 214 and Asn for Tyr at position 217;
c. Glu for Ala at position 216 and Leu for Tyr at position 217;
d. Leu for Ile at position 205 and Glu for Ala at position 216;
e. Leu for Ile at position 205 and Asp for Ala at position 216;
f. Glu for Gln at position 206 and Glu for Ala at position 216;
g. Asp for Ala at position 216 and Leu for Try at position 217; or
h. Glu for Gln at position 206 and Leu for Try at position 217.

9. The fabric cleaning composition of Claim 7, characterized in that

a. when a substitution occurs at position 206, the substituting amino acid for position 206 is Glu, Asn or Ser;
b. when a substitution occurs at position 210, the substituting amino acid for position 210 is Gly, Gln, Asn, Ser, Asp or Glu;
c. when a substitution occurs at position 211, the substituting amino acid for position 211 is Pro, Gln, Asn, Ser, Asp or Glu;
d. when a substitution occurs at position 214, the substituting amino acid for position 214 is Leu, Ile, Val, Met, Cys, Ala, His, Pro, Gly, Gln, Asn, Asp or Glu; and
e. when a substitution occurs at position 215, the substituting amino acid for position 215 is Pro, Gln, Asn, Ser, Asp or Glu.

10. The fabric cleaning composition of Claim 6, characterized in that when a substitution occurs at positions 199, 200, 201, 202, 203, 204, 205, 206, 207, 208, 209, 210, 211, 212, 214, 215, 216, 217, 218, 219 or 220, the substituting amino acid for any of positions 199, 200, 201, 202, 203, 204, 205, 206, 207, 208, 209, 210, 211, 212, 214, 215, 216, 217, 218, 219 or 220 is Asp or Glu; and when a substitution occurs at position 213, the substituting amino acid for position 213 is Asp; and wherein a substitution preferably occurs at two or more of positions 199, 200, 201, 202, 205, 207, 208, 209, 210, 211, 212, or 215, more preferably a substitution occurs at two or more of positions 200, 201, 202, 205 or 207.

11. The fabric cleaning composition of Claim 6, characterized in that Glu or Asp is substituted for Ala at position 216 and Leu is substituted for Tyr at position 217.

12. The fabric cleaning composition of Claim 6, characterized in that the wild-type BPN' is substituted at three positions.

13. The fabric cleaning composition of Claim 12 wherein the three substitutions are:

a. Pro substituted for Gln at position 206, Ala substituted for Gly at position 211, and Glu substituted for Ala at position 216;
b. Val substituted for Ile at position 205, Ala substituted for Pro at position 210, and Glu substituted for Lys at position 213; or
c. Glu substituted for Gln at position 206, Glu substituted for Ala at position 216, and Leu for Tyr at position 217.

14. The fabric cleaning composition of Claim 12 characterized in that

a. when a substitution occurs at position 206, the substituting amino acid for position 206 is Asn or Ser;
b. when a substitution occurs at position 210, the substituting amino acid for position 210 is Gly, Gln, Asn, Ser, Asp or Glu;
c. when a substitution occurs at position 211, the substituting amino acid for position 211 is Pro, Gln, Asn, Ser, Asp or Glu;
d. when a substitution occurs at position 214, the substituting amino acid for position 214 is Leu, Ile, Val, Met, Cys, Ala, His, Pro, Gly, Gln, Asn, Asp or Glu; and

e. when a substitution occurs at position 215, the substituting amino acid for position 215 is Pro, Gln, Asn, Ser, Asp or Glu.

15. The fabric cleaning composition of Claim 6, characterized in that the wild-type BPN' is substituted at four positions or five positions.

16. The fabric cleaning composition of Claim 15, wherein the substitutions are:

a. Glu substituted for Val at position 203, Glu substituted for Gln at position 206, Glu substituted for Ala at position 216, and Leu substituted for Tyr at position 217;
b. Glu substituted for Val at position 203, Ala substituted for Pro at position 210, Glu substituted for Ala at position 216, and Leu substituted for Tyr at position 217;
c. Glu substituted for Val at position 203, Glu substituted for Gln at position 206, Thr substituted for Gly at position 215, Glu substituted for Ala at position 216, and Leu substituted for Tyr at position 217; or
d. Glu substituted for Val at position 203, Ala substituted for Pro at position 210, Thr substituted for Gly at position 215, Glu substituted for Ala at position 216, and Leu substituted for Tyr at position 217.

17. The fabric cleaning composition of any of Claims 1 through 16, characterized in that said composition is in the form of a liquid.

18. The fabric cleaning composition of any of Claims 1 through 16, wherein the composition comprises at least about 5% surfactant and at least about 5% builder, by weight of the composition.

19. A method for cleaning fabric, said method comprising contacting a fabric in need of cleaning with the composition of any of Claims 1 through 18.

INTERNATIONAL SEARCH REPORT

Interna[illegible] Application No
PCT/US 95/04691

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C11D3/386 //C12N9/54

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C11D

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO-A-94 02618 (GIST-BROCADES) 3 February 1994 see the whole document especially Table II ~~~	1,6,7,9, 10,12, 15,17-19
X	WO-A-89 09819 (GENEX CORPORATION) 19 October 1989 see claims 1,3-5,9-11 ~~~	6,7, 17-19
X	WO-A-92 11357 (NOVO NORDISK) 9 July 1992 see claim 25 ~~~	1,17-19
X	EP-A-0 405 901 (UNILEVER) 2 January 1991 see claims 1-3,8 ~~~ ~/~~	6,7,12, 17-19

[X] Further documents are listed in the continuation of box C. [X] Patent family members are listed in annex.

* Special categories of cited documents :

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
27 July 1995	16.08.95

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Van der Schaaf, C

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INTERNATIONAL SEARCH REPORT

Interna... Application No
PCT/US 95/04691

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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A	CHEMICAL ABSTRACTS, vol. 116, no. 23, 8 June 1992 Columbus, Ohio, US; abstract no. 230623, P. BRODE AND D. RAUCH 'Subtilisin BPN': activity on an immobilized substrate' see abstract & LANGMUIR, vol. 8, no. 5, 1992 pages 1325-1329, cited in the application	
P,X	WO-A-95 07991 (THE PROCTER & GAMBLE COMPANY) 23 March 1995 see the whole document	1-19

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